SYNOPSIS

Ph.D in Food Science and Nutrition

DEVELOPMENT AND EVALUATION OF *ULVA LACTUCA* BASED PROBIOTIC BEVERAGE AND *IN VITRO* BIOAVAILABILITY OF IRON USING CACO-2 CELL MODEL

By

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a) Title of the Thesis:

Development and Evaluation of *Ulva lactuca* based Probiotic Beverage and *in vitro* Bioavailability of Iron using Caco-2 Cell Model.

b) Brief Description on the State of the Art of the Research Topic:

Iron deficiency Anaemia is a public health problem with significant implications for human health, affecting millions globally. Recent research underscores the critical role of iron in various physiological functions, including oxygen transport, DNA synthesis, and energy metabolism. Iron deficiency being the most common nutritional disorder worldwide, can lead to Iron Deficiency Anemia (IDA), characterized by fatigue, weakness, and impaired cognitive function (World Health Organization, 2021). Prado and Dewey (2018) demonstrated that iron deficiency in early childhood is associated with long-term cognitive deficits and altered brain structure. Maternal iron deficiency during pregnancy can lead to developmental delays and neurocognitive impairments in offspring. Iron deficiency also affects adult populations, impairing physical performance and productivity (Haas *et al.*, 2016). Mc Clung *et al* (2019), reported that iron supplementation improves physical performance in iron-deficient individuals. Petry *et al* (2016) highlighted the association between iron deficiency and increased susceptibility to infections, due to impaired immune function, emphasizing the role of iron in maintaining immune homeostasis. Given these multifaceted impacts, addressing iron deficiency through dietary interventions and public health strategies is crucial. Iron deficiency remains a critical public health challenge with wide-ranging effects on human health. Ongoing research and intervention strategies are essential to mitigate the adverse outcomes associated with this condition.

c) Definition of the Problem:

To combat Iron Deficiency Anemia in India, strategies involving dietary approaches to enhance iron bioavailability have been suggested. The pivotal role of gut microbiota in maintaining health and preventing a multitude of diseases has been extensively studied over decades. The societal and economic impact of IDA on developing nations is substantial, with estimated productivity losses amounting to 1.3% of India's Gross Domestic Product (FAO 2021). The intricate interplay between these beneficial microorganisms and the host has been investigated, revealing their profound impact on various physiological processes, immunomodulation, and disease prevention. Numerous initiatives have been launched to address IDA in India, like The National Iron Plus Initiative (NIPI), Anaemia Mukt Bharat

(AMB) and many more for combating Iron deficiency from the root level. However, their effectiveness has been hindered by administrative irregularities, gaps in program implementation, limited medical coverage, and societal unawareness rooted in cultural practices and beliefs (Sharma *et al.*, 2003).

Biofortification strategies have also emerged as a focal point in enhancing iron bioavailability in staple crops. Research by Li *et al* (2022) and Shubham *et al* (2020) investigated the effectiveness of genetic and agronomic interventions in increasing iron content and its bioavailability, thereby addressing micronutrient deficiencies in vulnerable populations. There is always a need to incorporate simple nutrient-dense ingredients in combinations to enhance nutrient bioaccessibility, especially iron, in daily diets.

d) Objectives and Scope of Research Work:

Primary Objective

To study the *in vitro* iron bioavailability of seaweed-incorporated probiotic beverage using the Caco-2 Cell Model and to determine whether its enhanced iron bioavailability shall serve as a natural food alternative for iron deficiency.

Specific Objectives: To

- Study the Nutrient and Heavy metal Composition of the selected seaweeds.
- Formulate and standardize the Seaweed-incorporated Probiotic Beverage.
- Determine the Nutrient profile, Probiotic potential, and Shelf life of the developed beverage.
- Assess the *in vitro* bioavailability of iron from the developed beverage using the Caco-2 cell model.

Scope of the Study

There is a need for novel usage of iron-rich seaweeds as a prebiotic to complement probiotics for enhanced iron bioavailability. Inadequate information about iron bio-availability from seaweeds is one of the factors that need to be addressed through this investigation. Prevalence of ambivalent research about enhanced iron bioavailability with probiotics in diet, could be addressed and there is a need for the development of novel food products with ingredients that are nutrient-dense, which is easy to prepare and consume. This investigation is a designated attempt to extensively propagate the use of nutrient-rich edible seaweeds in Indian cuisine.

e) Methodology

Several research studies have quoted the health benefits of edible seaweeds and have used them extensively in developing recipes that entice the palette of global cuisines. Four underexploited edible seaweeds namely *Ulva lactuca*, *Ulva reticulata*, *Gracilaria edulis*, and *Sargassum polycystum* were collected from Ramanathapuram District in Tamil Nadu. The selected seaweed species are consumed occasionally by the local population. All the seaweeds are harvested during the time of pre- and post-monsoon seasons when the climate is favourable for their growth, in the months of September to December. The seaweeds were identified with the help of CMFRI bulletin No.41 (1987). The seaweeds were preserved in 5% formaldehyde as per the wet preservation method suggested by Baweja (2016), from the collection site until the sample is prepared for investigation. The selected seaweeds were washed thoroughly in seawater and then in tap water. The seaweeds were again washed in distilled water, the remaining water was drained, and the fresh seaweeds were dried in a cabinet drier at ~70°C , pulverized and sieved using 40 mesh. It is further used for nutrient analysis. Quantitative estimation of proximate nutrients including moisture content, ash content, total carbohydrates, protein, fat, and crude fibre of *Ulva lactuca*, *Ulva reticulata*, *Gracilaria edulis*, and *Sargassum polycystum* was analyzed in triplicates using standard estimation procedures given by Food Safety and Standards Authority of India, (FSSAI, 2016). Total Carbohydrates were assessed with the Anthrone method wherein, protein was estimated using the Lowry method. The fat content of the seaweeds was estimated by the Soxhlet method using Socs plus analyzer in petroleum ether (60°C - 80°C). The total, soluble, and insoluble dietary fibre content of the seaweed was estimated using the standard procedure given by the AOAC, (2011). Powdered seaweeds were ashed as a prerequisite for analysis of micronutrients namely iron, phosphorus, calcium, zinc, and Vitamin-C, selenium and β-carotene which were analysed by standard AOAC methods, by an Atomic Absorption Spectrophotometer (AAS). Heavy metal toxicity was analysed for the hydrocolloids extracted with a selected method using an Atomic Absorption Spectrophotometer (AAS) of model Thermo Scientific ICE 3000 Series equipped with SOLAAR software and graphite tube atomizer. The protocols observed were adopted from the study by Pan *et al* (2018) and heavy metals like mercury, cadmium, lead, chromium and arsenic were analyzed.

The preparation of the beverage consisted of ingredients like orange juice (40%), whey (35-45%), *Ulva lactuca* (variable) and palm jaggery powder (5%), which were procured from the local market. Whey was made by preparing curd from locally procured milk and straining

it through a muslin cloth. *Ulva lactuca* extract was prepared and the beverage was made by mixing all the ingredients in standardized proportions. Variation 1 (V_1) contained 10% extract and Variations 2 (V_2) and 3 (V_3) subsequently contained 15% and 20% of extracts. Standard beverage was made without adding *Ulva lactuca*. The sensory evaluation was conducted with a total of 34 qualified panel members. The quality parameters such as colour, taste, appearance, flavour, and overall acceptability were assessed using 9-point Hedonic Scale, ranging from 1= Dislike extremely and 9 = Like extremely. The formulated beverage was standardized by trials to obtain optimum sensory appeal. The variation with the highest overall score was considered the best-accepted variation and it was chosen for further analysis.

The physical properties of the probiotic beverage are assessed using Quality parameters like pH, titratable acidity, and viscosity using Lab Care Export Digital PH Meter LB-901 and Viscometer-Brookfield Model RVDI, USA was used to quantify viscosity. The percentage of lactic acid was used to determine titratable acidity, which was executed by titrating with 0.1 NaOH, with phenolphthalein as an indicator. Brix was measured using a Digital refractometer (Rudolph, USA). The proximate nutritional composition of the developed probiotic beverage was assessed using the protocols mentioned during the estimation of seaweeds. Dietary fibre was determined using the enzymatic and gravimetric - AOAC 985.29 method (AOAC, 1997). The Total Phenolics (TP) were determined according to the Folin-Ciocâlteu method adapted for microplate assay (Xue *et al.*, 2009). Total flavonoid (TF) content was estimated in the same extract used to determine TP and quantified using the method given by Kim *et al* (2003) for the spectrophotometry method. Alkaloids by Hager's test and phytosterol by Salkowski's test were carried out with the standard procedure of Samec *et al* (2016), for qualitative estimation. Overall Phytochemical Composite Index (OPCI) was devised based on the relative concentrations of four phytochemicals – total polyphenols, total flavonoids, total oxalates and alkaloids. Micronutrient and Anti-nutrient Quality Score (MQS) was determined by dividing the individual quantities of the nutrient by the respective RDA values multiplied by 100. The antioxidant activity of the beverage was measured based on the scavenging activity of the stable 1, 1- diphenyl 1- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the procedure as described by Brand-Williams *et al.*, (1995) with slight modifications. FRAP Assay was determined according to the microplate method described by Lister *et al* (2019). The developed probiotic beverage was assessed for the presence of bioactive compounds through GC-MS. Gas chromatographic

separations were achieved on a Thermo Scientific TRACE™ 1310 Gas Chromatograph with a single quadrupole mass spectrometer. Bioactive compounds with a match factor greater than eighty were documented and considered for further study.

The 2D structures of the identified phyto-compounds (ligand molecules) from the beverage through GC - MS analysis were drawn in ACD – Chemsketch (ACD/ ChemSketch Freeware, Version11, 2006) and their SMILES notation was obtained. The SMILES notation was submitted to online SMILES converter and structure file generator and further converted to 3D PDB file format (Weininger 1988). The obtained 3D PDB files and SMILE notations of ligands were utilized for further study (Thirumalaisamy *et al.*, 2018). The drug-likeness nature of the abundant phyto-compounds and *in-silico* ADME (Absorption, Distribution, Metabolism, and Excretion) profiling was analyzed using QikProp module (Version 3.2, 2009, Schrödinger, LLC) online server and examined based on the violations of Lipinski rules. The properties predicted were including log P (octanol/water), % human oral absorption in intestine (QP %) and predicted aquous solubility in log S (molar), blood brain barrier (BBB), human intestinal absorption, Caco-2 permeable, number of likely metabolic reactions, number of property/descriptor values that fall outside the 95% range of similar values for known drugs and molecular weight which determines oral bioavailability.

Isolation of the probiotic strain from the beverage was done using serial dilutions and streak plating on MRS agar plates. Physiological characterization of the strain was done by Gram staining and microscopic observation. Biochemical characterization was done using Endospore test, motility test, Indole test, Vogues-Proskauer, Methyl-red, catalase and Citrate Utilisation tests. Morphological Identification was done using DNA extraction, purification and PCR amplification. A BLAST search was used to match the obtained sequences to those in the NCBI repository. *In vitro* probiotic potential assessed the tolerance of the isolated strain to pH, simulated gastric juice, bile and pancreatic juice. The Surface-Hydrophobicity Index was calculated as per the protocol suggested by Reuben Roy *et al* (2020). NaCl tolerance and Auto Aggregation Assay were carried out as per the methods given by Krausova *et al.*, 2019. Antimicrobial and antibiotic activity and haemolytic activity was meticulously assessed. The microbial safety of the selected films was assessed using pH, titratable acidity, Total Viable Count (TVC), Total Bacterial Count and Total Fungal Count of the beverage with the standard method (Thakkar *et al.*, 2018).

In vitro digestion was performed according to the cell-free model described by Gangloff *et al* (1996) with modification described by Venkatasubramanian *et al* (2014).

The Caco-2 (Human colorectal adenocarcinoma cell line) was purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2. The developed probiotic beverage sample was tested for *in vitro* cytotoxicity, using Caco-2 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The test compound (Fe + V_1) and control dialysates (Fe/AA/ V_1 alone and blank) were used to study the iron uptake in Caco-2 cells. The amount of ferritin in cell lysate was estimated by Raybio Ferritin ELISA kit (Ray Biotech, Norcross, GA) following kit protocol and expressed in terms of µg/ml. All data was analyzed using SPSS v.17.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at $p< 0.01$ and $p<0.05$.

f) Original Contributions - Salient Findings of the Study:

The salient findings of the current study are summarised below:

- The morphological characteristics and taxonomy of the four edible underexploited seaweeds *Ulva lactuca*, *Ulva reticulata*, *Gracilaria edulis*, and *Sargassum polycystum* are classified as Chlorophyta, Rhodophyta and Phaeophyceae according to Fritsch *et al* 1935. The colour of the seaweeds ranged from light green to dark brown as per their species and all are found to be free-floating masses.
- *Ulva lactuca* showed 10.24±0.02 of percent moisture content, 20.62±0.06 g of ash, 25.81±0.12 g of total carbohydrates, 19.22±0.13 g of crude protein, 2.13±0.1 g of fat and 3.35±0.01 g of crude fibre content per 100g of the dry weight of seaweed. *Ulva reticulata* showed 10.54±0.05 percent of moisture content, 19.96±0.05 g of ash, 22.52±0.06 g of total carbohydrates, 22.10±0.07 g of crude protein, 2.18±0.02 g of fat and 4.54±0.18 g of crude fibre content per 100g of the dry weight of seaweed. *Gracilaria edulis* showed 8.39±0.04 percent of moisture content, 15.25±0.03 g of ash, 17.15±0.05 g of total carbohydrates, 20.35±0.05 g of crude protein, 3.27±0.04 g of fat and 3.92±0.04 g of crude fibre content per 100g of dry weight of seaweed. *Sargassum polycystum* showed 7.15±0.02 percent of moisture content, 9.14±0.01 g of ash, 19.43±0.04 g of total carbohydrates, 18.17±0.01 g of crude protein, 1.18±0.04 g of fat and 4.14±0.02 g of crude fibre content per 100g of the dry weight of seaweed.
- The heavy metal composition of the selected seaweeds revealed that Lead and Arsenic were found to be below detectable levels. Mercury, Nickel, Cadmium and Chromium

levels of *Ulva lactuca, Ulva reticulata, Gracilaria edulis* and *Sargassum polycystum* were found to be within the recommended safe limits.

- The sensory evaluation of the Standard and Variant 1, 2 and 3 were assessed for appearance, colour, consistency, flavour, taste and acceptance of the beverages. The organoleptic score of Standard beverage ranged from 8±0.56 to 8.65±0.58, across the parameters assessed. For variation 1, the score ranged from 6.95±0.51 to 7.2±0.52. Variant 2 demonstrated organoleptic scores of 6.15±0.48 to 6.15±0.48. Variant 3 ranged from 5.5±0.6 to 5.6±0.5.
- The physical characteristics of the developed probiotic beverage variants of Standard and Variant 1 demonstrated 6.95 and 7.82 pH respectively. The standard beverage showed 18.25±0.07 of Total Soluble Solids content, 0.09±0.02 Lactic acid equivalent of percentage titratable acidity, 736.6 Viscosity and a specific gravity of 1.24. On the other hand, Variant 1 demonstrated 18.78±0.03 of Total Soluble Solids content, 0.15±0.03 Lactic acid equivalent of percentage titratable acidity, 758.7 Viscosity and a specific gravity of 1.36.
- The proximate nutritional composition per 100 mL of the Standard beverage showed 6.7±0.3 g of Total carbohydrates, 0.75±1.23 mg of reducing sugars, 0.68±0.03 g of crude protein, 0.42±0.23 g of crude fat, 0.6±0.03 g of crude fibre, 0.1±0.02 g of dietary fibre, 187±2.52 kcal of energy, 8.3±0.07 g of Iron, 3.1±0.03 g of zinc, 1.9±0.02 g of calcium, 62.6±0.05 g of sodium, 112.7±0.09 g of potassium, 33.2±0.02 µg of β-carotene, 20.1±0.01 µg of Folic acid, 1.2±0.05 µg of Cyanocobalmin, 5.3±0.04 g of Ascorbic acid. The proximate nutritional composition per 100 mL of the Variant 1 of the beverage showed 6.8±0.04 g of Total carbohydrates, 0.68±0.89 mg of reducing sugars, 0.79±0.02 g of crude protein, 0.37±0.41 g of crude fat, 0.8±0.03 g of crude fibre, 0.2±0.02 g of dietary fibre, 192.81±3.1 kcal of energy, 12.8±0.07 g of Iron, 4.2±0.02 g of zinc, 2.2±0.04 g of calcium, 65.92±0.03 g of sodium, 121.4±0.05 g of potassium, 38.4±0.04 µg of β-carotene, 29±0.05 µg of Folic acid, 1.3±0.02 µg of Cyanocobalmin, 5.9±0.05 g of Ascorbic acid.
- Quantitative estimation of phytochemical profile of Standard beverage showed 1.8±0.02 mg of phytates, 10±0.03 mg of oxalates, 284±0.03 mg GAE of total phenolics, 655±0.05 mg QE of total flavonoids and 12.31±0.03 mg of alkaloids content. On the other hand, Variation 1 demonstrated 2.4±0.02 mg of phytates, 21.5±0.02 mg of oxalates, 292±0.03 mg GAE of total phenolics, 664±0.02 mg QE of total flavonoids and 13.14±0.03 mg of alkaloids content.

- The correlation analysis for appearance, colour, taste, flavour, acceptance, TPC, TFC and Ascorbic Acid revealed that very strong correlations were observed between taste and TPC and Colour and TFC, with $r = 0.998$, followed by Acceptance and TFC with $r = 0.978$; Flavour and Acceptance with $r = 0.977$. Appearance and Flavour had a strong correlation of $r = 0.972$. Ascorbic Acid and Taste; Ascorbic Acid and TFC and Colour and Taste had a similar correlation coefficient of $r = 0.962$. Strong correlations were also observed between Ascorbic Acid and TPC with $r = 0.954$. TFC and TPC and TFC and Flavour correlated with $r = 0.949$. Appearance and Colour and Colour and Flavour demonstrated an $r = 0.927$ and 0.928 respectively. A strong correlation coefficient of $r = 0.896$ was observed between acceptance and TPC. A correlation coefficient ranging from $r = 0.893$-0.839 was observed between various combinations of sensory attributes and phytochemicals. All the values obtained were significant at $p \leq 0.01$ and $p \leq 0.05$.
- The individual phytochemical indices of the standard probiotic beverage were calculated with respect to Variant 1 capped at 100. The Overall Phytochemical Composite Index (OPCI) of the standard beverage was calculated to be 94.89 and hence could be consumed at par with the *Ulva lactuca*-based probiotic beverage (V_1).
- A total of seven micronutrients and two anti-nutrients were assessed to determine the % RDA contribution of both beverage variants. It could be noted that 85.33% of RDA is met by Variant 1 whereas 55.33 % by Standard beverage. The difference in the values could be attributed to the use of *Ulva Lactuca* in Variant 1. The RDA of Vitamin B 12 was 65% met by V1 when compared to Standard beverage. 38.18% daily requirement of zinc was met by variant 1 when compared to the standard beverage. A lower dietary requirement of Vitamin C was met by variant 1 viz. 9.08% and 8.15% by Standard beverage. Both the beverages proved to be a poor source of calcium by contributing to only 0.24-0.28 % RDA. 4.67% and 4.33% of Potassium RDA requirements were met respectively by Variant 1 and Standard beverage. Phytates and oxalates were estimated to be 2.40% and 14.33% for Variant 1 and 1.80% and 6.67% respectively for the standard beverage. As per National Institute of Nutrition nutrient content claims, food is categorised as a good source, if it contains 10-19 % of the Dietary Values of proteins/ vitamins/ minerals or dietary fibre.
- The DPPH Radical Scavenging Assay revealed that both Standard and Variant 1 of the developed probiotic beverage have shown very strong scavenging activity against DPPH, evident from the graph. Variant 1 showed an exceptionally high DPPH RSA

with an IC_{50} value of 5.4 µg/mL, which is higher than Ascorbic acid, with an IC_{50} value of 6.01 µg/mL, which was taken as a positive control for the study. The Standard beverage exhibited an IC50 value of 18.42 µg/mL, which is also commendable. With regard to the exceptional IC50 values demonstrated by the beverage, when treated with DPPH, it can be considered a very good source of antioxidants. Variant 1 was found to exhibit 95.33±2 µmol Fe/g radical scavenging activity when compared to Standard, with 91.8±2.3 µmol Fe/g Radical scavenging activity.

- Pearson's correlation between total phenolics, total flavonoids, total alkaloids, and antioxidant activities revealed that very strong correlations were observed between total flavonoids and DPPH RSA with $r = 0.985$, followed by total flavonoids and FRAP with $r = 0.984$ and total phenolics and total flavonoids with $r = 0.949$ and FRAP and DPPH RSA with $r = 0.940$ at $p \leq 0.01$. Strong correlations with $r > 0.8$ were also observed between total phenolics and DPPH RSA with $r = 0.833$ at $p \leq 0.01$. Negative correlation was observed between alkaloids and DPPH RSA with $r = -0.835$. No correlation was observed between Alkaloids, Antioxidant assays and other phytochemicals. Hence, the role of phenolics and flavonoids in the antioxidant activities of the developed probiotic beverage was confirmed.
- Gc-MS profiling of bioactive compounds present in Variant 1 of the developed probiotic beverage revealed a total of 57 compounds were identified through GC-MS/MS out of which 13 compounds exhibited a match factor of >80% similarity with those established in the database. Seven bioactive compounds exhibited abundance at retention times of 3.7898, 13.2937, 18.6945, 22.2434, 24.9704, 27.3664 and 29.6566. These were identified as propionic acid, ethyl decanoate, octadecenamide, heptacosane, methyl hexadecanol, squalene and octadecanoic acid methyl ester. These bioactive compounds were found to have therapeutic properties and were found to play a crucial role as antioxidant, anti-carcinogenic, ant-pyretic, anti-inflammatory, antibacterial, anti-allergic and also in ATPase generation.
- *In-silico* ADME (Absorption, Distribution, Metabolism, and Excretion) profiling was performed to assess the pharmacokinetics properties for the selected bioactive compounds. Octanol/Water Partition Coefficient (Log P), indicates lipophilicity. Values between 1 and 3 are typically favorable for oral drugs. The compounds exhibited values between 3.829 in Squalene to 7.806 in octadecenamide, indicating moderate favourability. The percentage oral absorption indicated very good absorbability ranging from 80.359% in propionic acid, to 100% in ethyl decanoate,

heptacosane, methyl hexadecanol, squalene and octadecanoic methyl ester. High permeability was predicted to be observed in Caco-2 cell model, with the log values ranging from log values 294.56 in octadecenamide to log value 9906.03 in squalene and heptacosane. Solubility (Log S) depicts the logarithm of the aqueous solubility and higher solubility (values closer to 0 or positive) is generally favourable for oral drugs. Propionic acid showed highest log value with 0.027 and low log values were observed for other compounds. Lipinski Rule of Five complied by all the abundant bioactive compounds and these compounds typically have optimal solubility and permeability, ensuring effective absorption and bioavailability. Compliance with these rules often results in better pharmacokinetic profiles, enhancing their potential as orally active drugs.

- The current study reported the presence of small, and clustered creamy white and shining colonies of bacteria with a mucilaginous appearance on the superficial plane of the agar medium. The shape of isolates under the microscope (100x oil immersion objective) revealed long rod-shaped bacilli, confirming the gram-positive bacteria. isolates exhibited negative outcomes for the tests' indole, Voges-Proskauer, methyl-red, endospore production, oxidase, catalase, and citrate utilization. Catalase negative bespeaks the ability of the isolates to not produce catalase enzyme (Akinola and Osundahunsi 2017). The isolates did not produce spores and showed negative for the catalase test. The hanging drop method showed the non-motility of the bacteria. It is one of the distinctive features of *Lactobacillus* where the flagella are absent. the isolate was capable of fermenting glucose, lactose, sucrose, fructose, maltose, galactose, and ribose.
- The generated BLAST results demonstrated that the 16S rDNA sequence of the isolated strain KYK demonstrates a high degree of similarity with species of *Lactobacillus* indexed in the GenBank and archived the accession number OP389067. The isolates showed the highest homology of 99% similarity index regarding species bacterial genera *Lactobacillus reuteri and Limosilactobacillus reuteri.*
- The *in vitro* probiotic potential of the isolate OP389067 showed analogous growth at pH 3.0 when collated with pH 7.2. there is a statistical association between the variables *L. reuteri* at pH 3.0 and pH 7±0.2 as the significance is 0.000, which is statistically significant (at 1% level). From the mean value, it is noted that sample Assay demonstrated more pH tolerance (41.70%) when compared to control samples.

L. reuteri isolate OP389067 at 3 pH when compared to the analogous increase in the growth of the organism at neutral pH.

- *L. reuteri* isolate OP389067 showed a survival rate of 99.4% after first-hour incubation. The viability decreased to 98.76% in the second hour and a marginal decrease was observed in the third hour, which is 98.25%. Independent sample t-test results revealed that there was a statistical association established between the variables as the significance (p-value) value is 0.000, it was statistically significant (at a 1% level). From the mean value it is noted that *L. reuteri* isolate OP389067 at 3 pH demonstrated significant Simulated Gastric Juice Tolerance when compared to control at pH 7.2 without Gastric juice.
- The bile juice tolerance of the isolate OP389067 showed the statistical association between *L. reuteri* (3pH) and *L. reuteri* (7.2pH) presented that there is a significant association between the variables at 5% level, as the significance (p-value) value is 0.019. From the mean value, it is noted that *L. reuteri* with 0.3% Bile at pH 8 had more Bile Tolerance when compared to *L. reuteri* control. The *L. reuteri* isolate OP389067 showed a survival rate of 10.15% during 0-4h and with relation to absorbance, the isolate demonstrated a survival rate of 10.63% at 3 h, and there was considerable growth after 4h incubation time.
- The pancreatin tolerance of the *L. reuteri* isolate OP389067 showed a statistical association between *L. reuteri* isolate OP389067 at 0.5% bile concentration and the control illustrated a 5% significance as the significance (*p*-value) value is 0.015. It was observed that there was a remarkable growth in the isolate at 24h in the presence of 0.5 percent pancreatin and the viability index of *L. reuteri* isolate OP389067 is calculated to be 25.33% at 24 h and 10.22% after 48 h incubation.
- The potentiality of *L. reuteri* isolate OP389067 was determined by its ability to adhere to the intestinal epithelial cells. The isolate exhibited a remarkable 72.22% adhesion for the n-hexadecane hydrocarbon and also a significant 54.4% auto-aggregation thus exhibiting the potential to colonize and adhere to the intestinal epithelium. The rate of NaCl tolerance of *L. reuteri* isolate OP389067 is inversely proportional to the concentration of NaCl over 12 h duration. Considerable growth of the isolate was observed until 6.5% concentration of NaCl and thereby the absence of growth at and beyond 7.8% NaCl concentration, where tolerance to high salt conditions (6 - 8 percent) has been reported to be a distinctive feature in most species of *Lactobacillus* species.

- *L. reuteri* isolate OP389067 showed γ-haemolysis or no haemolysis, which is a desirable characteristic of beneficial probiotic bacteria aiding in gut health. *L. reuteri* isolate OP389067 was found to be resistant to Methicillin – MET 5mcg and Ceftazidime – Cz 30mcg (Table 3). The association between the isolate and the susceptibility towards antibiotics was analyzed through One-Way ANOVA and statistical association between the variables as the significance (p-value) value is 0.000, is established at 1% level. The antagonistic activity of *L. reuteri* isolate OP389067 and its antibacterial potential was assessed against *S.aureus, P.aeruginosa, B.cereus, S.typhi,* and *E.coli,* which are the most common enteropathogens affecting gut health. The isolate demonstrated moderate sensitivity towards *B.cereus* and *S.aureus* with a zone of inhibition of 10±0.01 and 11±0.01 respectively. Very low sensitivity was observed for *P.aeruginosa, S.typhi,* and *E.coli* with a zone of inhibition 8±0.05, and 9±0.02 towards both organisms respectively. The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism. The isolate exhibited a MIC of 1.5, 1.6, 0.8, 0.9 and 1.2 mg/mL by *S. aureus, E. coli, K. pneumoniae* and *B. cereus* respectively.
- The shelf-life study data of the developed probiotic beverage revealed that the cell viability of *L. reuteri* isolate OP389067 ranged from $3.36x10^7$ to $6.92x10^8$ which justifies the standard viable count of bacteria essential to cater to the therapeutic benefits of the developed probiotic beverage. The shelf-life study of the beverage at a refrigerator temperature of 4°C recorded the absence of fungus from0-6^{th} day of storage duration. From day 8^{th}-10^{th}, the fungal count was found to be below the detectable level for the standard beverage and the Variant 1. The total bacterial count for both standard product and the variant was found to be exponentially increasing from $3.27x10^7$ to $6.83x10^8$ and $4.76x10^7$ to $6.70x10^8$ for the standard and variant respectively. The pH of the product gradually decreased from the range of 7.0 to 5.0 for both standard and variant beverages within 10 days duration. The percentage acidity showed a spike in values for both standard and variant beverages for a span of 10 days. From the above data, it was noted that the shelf life of the studies of probiotic beverages containing *U. lactuca* at 4±2°C was found to be best for consumption within 10 days of preparation at a refrigerator temperature of 4±°C.
- *In vitro* cytotoxicity study of the developed probiotic beverage using Caco2 cell lines was done using MTT Assay. It was observed that the cell lines had 84.2 % viability

when supplemented with control samples of the beverage. The viability of cell lines for the developed probiotic beverage was found to be 84.2%. The decrease in cell viability indicates that the cell lines did not proliferate after supplementation with the beverage sample. The decrease in proliferation of cell lines was due to the protective action of Ascorbic acid present in the beverage samples.

- In the current study, a low content of Ferritin was observed in individual groups, and it was enhanced with the combination of Iron. Iron in combination with 50 µg of Ascorbic acid showed 168.26±1.42 µg/ml of ferritin uptake, followed by 150.88±5.73 µg/ml of ferritin uptake by 50 µg of Variant 1 in combination with ascorbic acid. 50 µg of Iron and V_1 showed 145±3.45 µg/ml of ferritin uptake and 50 µg of Variant 1 showed 128.62±0.70 µg/ml. The untreated cells showed 11.06±0.67 µg/ml and 2.52±0.47 µg/ml respectively. Effective Iron uptake was scored in Ascorbic acid and Sample V_1 with 50ug/ml concentration in Caco-2 cells and confirmed that Sample V_1 have the potential of Iron bioavailability in the Human intestinal model.
- Cellular Protein concentration observed in different culture conditions of Caco-2 cells revealed that 50µg of Iron with Ascorbic Acid has shown a protein concentration of 49.24±1.72 µg/mL, followed by V1 in combination with ascorbic acid, which showed a protein concentration of 45.52±0.19 µg/mL. 50µg of Iron in combination with Variant 1 of the beverage demonstrated a protein concentration of 38.52±0.15 µg/mL, followed by Standard iron solution, Variant 1 and untreated samples have demonstrated a protein concentration value of 27.60±1.14 µg/mL, 25.02±0.88 µg/mL and 20.71±1.51 µg/mL respectively.
- The iron uptake study in Caco-2 cell model recorded that iron bioavailability has increased more than 10 times in 50 ug of Variant 1 of the beverage. In the presence of 50ug of Ascorbic acid, 15 times increase of percentage iron bioavailability was observed and, when V_1 in the presence of Ascorbic acid showed a 13-fold increase in percent bioavailability of iron.

g) Papers Published:

- Karishma Yadav. G, and Kowsalya. S., (2022). Formulation and Evaluation of Nutritional and Bioactive Compounds in a Probiotic Beverage Containing *Ulva Lactuca*. *The Indian Journal of Nutrition and Dietetics*, *59*(4), 467–477. https://doi.org/10.21048/IJND.2022.59.4.31320.

- Karishma Yadav. G, and Kowsalya. S., (2024). Probiotic properties and safety assessment of *lactobacillus reuteri* in a beverage containing *Ulva sp. The Indian Journal of Home Science*, 36(1), 330-341.

h) Conclusion:

Ulva lactuca showed to be a good source of minerals with ash content of 20.62 mg/100 g dry wt. The iron (168 mg/100 ml) and Vitamin C (46mg) in *U. lactuca* proved to be good food alternative for Iron Deficiency. The developed probiotic beverage contained 12.08 mg of Iron, 38.4 µg of β-carotene, 29 mg of Vitamin B9 and 5.9 mg of ascorbic acid. The nutraceutical profile of the developed beverage portrayed 68 mg TPC, 658 mg TFC, 128 mg of Alkaloids, ascerting it to be a good souce of bioactive compounds. The beverage showed good Radical Scavenging Activity when analysed by DPPH and FRAP methods. Strong correlation was observed between sensory attributes and selected phytochemicals in determining the sensory attributes of the developed probiotic beverage. The Overall Phytochemical Composite Index (OPCI) of the standard beverage was calculated to be 94.89 and hence could be consumed at par with the *Ulva lactuca* based probiotic beverage (V_1). The beverages also exhibited appreciable micronutrient and antinutrient quality scores.

GC-MS study for bioactive compounds revealed 30 innate bioactive compounds in which several aldehyde, ketone and alcohol derivative groups are found to possess good anti-cancer, anti-fungal, anti-bacterial activities. Seven bioactive compounds exhibited abundance at retention times of 3.7898, 13.2937, 18.6945, 22.2434, 24.9704, 27.3664 and 29.6566. These were identified as propionic acid, ethyl decanoate, octadecenamide, heptacosane, methyl hexadecanol, squalene and octadecanoic acid methyl ester. *L. reuteri* isolate OP 389067 proved to have good probiotic potential effective and safe for consumption.

Cytotoxicity study of Caco-2 cells revealed negligible toxicity levels hence proving the beverage to be safe for consumption. The iron uptake in *in vitro* Caco-2 cell model showed that when treated with 50µg of beverage, 128.62 µg/ml of ferritin concentration was observed. Good results were shown when 50µg of the beverage was treated along with Ascorbic acid (150.88 µg/ml ferritin conc.), which is comparable to 168.26 µg/ml of ferritin conc. Showed with standard Iron treated with Ascorbic acid. Cellular protein concentration was found to be 45.52 µg/ml in 50µg of the beverage was treated along with Ascorbic acid when compared to standard Iron treated with Ascorbic acid. The *U. lactuca* based probiotic beverage proved to be a good food alternative to be considered in alleviating the symptoms of Iron Deficiency Anemia (IDA).

From the foregoing results, it is evident that it was feasible to develop a probiotic beverage incorporating *Ulva Lactuca*. The beverage was nutrient-rich and the probiotic strain *L.reuteri* OP389067 demonstrated probiotic potentials in terms of tolerance towards pH, simulated gastric juice, bile juice and pancreatic juice. Also, the isolate demonstrated antibiotic susceptibility and activity against common food-borne bacteria. The nutrient and nutraceutical potentials of the developed probiotic beverage showed prominent antioxidant properties. The beverage was also rich in bioactive compounds, catering to the therapeutic attributes of the beverage. The *in vitro* bioavailability study of Iron using the Caco-2 cells demonstrated that the beverage showed good ferritin uptake in the presence of Ascorbic acid, and hence showed to be a good source of bioavailable iron.

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